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Personalising and targeting antiangiogenic resistance: a complex and multifactorial approach

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Pathological angiogenesis involves complex and dynamic interactions between tumour cells and other lineages existing in the microenvironment of the tumour. Preclinical and clinical data suggest that tumours can show dual, different adaptive responses against antiangiogenic agents: one successful adaptation is vascular normalisation, whereas the second adaptation is elicited through vascular trimming and increased hypoxia. These phenomena depend on the type of tumour and the type of agent. The classical approach for investigating acquired resistance against antiangiogenic agents is to identify compensatory signalling pathways emerging in response to VEGF blockade, which has led to the development of highly effective drugs; however, ultimately these drugs fail. Here we review how the dual stromal adaptive patterns determine the mechanisms of escape that go beyond the reprogramming of signal transduction pathways, which obliges us to investigate the tumour as an ecosystem and to develop uni- and multicompartmental models that explain drug resistance involving metabolic and immune reprogramming. We also propose a method for facilitating personalised therapeutic decisions, which uses 18F-fluoromisonidazole-positron emission tomography to monitor the dual stromal response in tumours of individual patients.

Under physiological conditions, angiogenesis is tightly regulated, and leads to a mature, well-constructed vascular network. By contrast, the normal regulation of angiogenesis fails under pathologic conditions such as cancer, and aberrant formation of blood vessels frequently occurs. This structurally abnormal network leads to aberrant local blood flow, fluid dynamics, and oxygenation, resulting in tumour growth and local and metastatic invasion by cancer cells (Jain, 2013). Research on the understanding of the process of angiogenesis has evolved considerably since Judah Folkman first introduced the hypothesis of the 'diffusion limit of oxygen' (Folkman, 1971). Considering the essential role of angiogenesis in tumour growth and progression, Folkman proposed the targeting of angiogenesis signalling molecules as a new approach for treating human cancer (Folkman, 1990). Since then, considerable effort has gone into the development of antiangiogenic drugs, and a number of these inhibitory agents have been approved by the FDA for clinical use against several types of cancer. However, tumours often escape the effects of these agents, and the disease eventually progresses. Elucidating the molecular mechanisms underlying the adaptive tumour response to antiangiogenic agents is a major need in clinical oncology.

RESISTANCE AGAINST ANTIANGIOGENIC AGENTS: A CLASSIC SIGNALLING PERSPECTIVE

Traditionally, the study of antiangiogenic resistance has focused on the vascular endothelial growth factor (VEGF) axis, including the proangiogenic driver VEGF-A and its receptor VEGFR-2. The development of antiangiogenic drugs has focused for decades on the inhibition of VEGF. The first agent receiving FDA approval was bevacizumab, a humanised monoclonal antibody against VEGF-A; bevacizumab administered in combination with chemotherapy increased the overall survival of colorectal cancer patients by 5 months (Hurwitz et al, 2004). Multitargeted tyrosine kinase inhibitors (TKIs) target the kinase domains of the tyrosine kinase

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Published by Springer Nature on behalf of Cancer Research UK.

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VEGF receptors and other tyrosine kinase receptors. Among the TKIs, sorafenib and sunitinib have been tested most extensively in phase III trials, showing positive results in liver and kidney cancers. The failure of this therapeutic approach due to acquired resistance has led to the increased understanding of VEGF-independent angiogenesis. Chronic VEGF/VEGFR2 inhibition was found to lead to the emergence of specific compensatory signalling pathways that sustain angiogenesis. These pathways rely on key proteins, including fibroblast growth factor (FGF) and its receptors (FGFR; Pepper *et al*, 1992), angiopoietin-2 (Ang2; Oliner *et al*, 2004), and the *MET* oncogene (Lu *et al*, 2012). These signalling pathways have been shown to regulate angiogenesis and serve as alternative inducers of tumour growth. Over past decades, complementary therapies that inhibit angiogenesis indirectly have been developed in attempts to abrogate acquired resistance. Targeting the Ang-Tie axis was proven successful, at least for ovarian cancer, where trebananib, a peptibody that inhibits the binding of Ang1/2 to their receptor, Tie2, was granted FDA approval in lieu of a phase III trial (Monk *et al*, 2014). Similarly, cabozantinib, an anti-MET agent, was successful in a phase III trial of patients with kidney cancer who had been previously exposed to anti-VEGFR2 therapy (Choueiri *et al*, 2015). These results support the theory that these axes are involved in compensatory responses against chronic VEGFR2 blockade. Unfortunately, despite these new approaches, treatment failure ultimately occurs in every patient with metastatic cancer. The obvious conclusions are that different mechanisms account for the perpetuation of pathologic angiogenesis, and that acquired resistance requires other approaches beyond the inhibition of signal transduction. Evidence increasingly points to tumour microenvironment and interactions between tumour cells and non-malignant cells as factors involved in the adaptive response of cancers against antiangiogenic agents. Thus, the mechanism of resistance to antiangiogenic therapies must be thought of as a dynamic multifactorial process. Integrating the inhibition of signalling pathways with the manipulation of the dynamic changes in tumour microenvironment and stromal cell interactions may optimise the inhibition of angiogenesis and overcome resistance, therefore improving patient outcomes (Figure 1).

DUAL PATTERN OF MICROENVIRONMENTAL RESPONSE AND RESISTANCE AGAINST ANTIANGIOGENIC THERAPIES

For years, 'vascular normalisation' has been assumed to be the general mechanism of action of antiangiogenic agents (Jain, 2013). The 'normality' concept applies not only to the blood vessels and their structure but also to the intercellular matrix, the type and polarisation of leukocytes, and fibroblasts (Huang *et al*, 2012; Jain, 2013). However, sufficient available preclinical and clinical data strongly indicate that antiangiogenic treatment is not always followed by vascular normalisation, and that tumours can manifest dual, different adaptive responses against antiangiogenic agents. One successful adaptation is vascular normalisation (followed by hypoxia correction), whereas the second adaptation is elicited through vascular pruning followed by increased hypoxia (Sennino and McDonald, 2012). The type of response would be determined by the type of agent, dynamic changes in the concentrations of pro- and antiangiogenic factors within the tumour microenvironment (and thus the timing of treatment administrations), and tumour type. The heterogeneity of adaptive responses to antiangiogenic therapy has implications for tumour metabolic states and the distribution of infiltrating immune cells infiltrate, which, in turn, impact the mechanisms of acquired resistance (Figure 2).

One study of patients with lung cancer who received radiolabelled docetaxel and underwent PET found that bevacizumab reduced both the perfusion and the net influx rate of docetaxel into the tumour, which contradicts the theoretical effects of normalisation (Van der Veldt *et al*, 2012). Similar controversial results have been found for sunitinib and sorafenib. Using electron paramagnetic resonance imaging of mice with squamous cell carcinoma xenografts, Matsumoto *et al* (2011) found that sunitinib normalised vasculature and tumour oxygenation. However, other investigators have found that sunitinib increased hypoxia, and that this effect was associated with epithelial-to-mesenchymal transition, stimulation of cancer stem cells, and metastases (Cooke *et al*, 2012). Similarly, results of other studies have shown this dual response to sorafenib (Murphy *et al*, 2006; Kumar *et al*, 2007; Liu *et al*, 2012). Taken together, these data suggest that responses to antiangiogenics include either decreased or increased tumour hypoxia, and that these responses might be tumour-dependent and agent-dependent. We sought to clarify these suppositions. In one experiment, we explored the 'tumour variable' and found that the same TKI (dovitinib) administered to two different pancreatic cancer tumourgraft models induced vascular and hypoxia normalisation in one model (followed by improved delivery of interstitial chemotherapy), while the opposite effects were observed in the other one (Hernandez-Agudo *et al*, 2016). In a second experiment, we explored the 'agent variable' and tested several multi-TKIs (nintedanib, dovitinib, and axitinib) and a monoclonal antibody (B20-4.1.1) on the same tumour model (MMTV-PyMT). We found that both axitinib and B20-4.1.1 increased hypoxia, while the other agents corrected it (Navarro *et al*, 2016). Given the unpredictable responses (whether or not an antiangiogenic agent will normalise a specific tumour), we realised that identifying which of the two possible stromal responses was occurring in an individual patient was highly relevant. Accordingly, we demonstrated that the vascular normalisation response could be monitored by a non-invasive test (PET using 18F-fluoromisonidazole, a probe that binds to tissues with <1% oxygen) (Hernandez-Agudo *et al*, 2016). Interestingly, these findings were validated in the clinical setting, where 18F-fluoromisonidazole-positron emission tomography (18F-MISO-PET) demonstrated that patients undergoing neoadjuvant treatment with nintedanib could show both increases or normalisation of hypoxia, and that the pattern of hypoxia correction was correlated with response (Quintela-Fandino *et al*, 2016).

ANTIANGIOGENIC RESISTANCE IN THE CONTEXT OF NORMALISATION: UNICOMPARTMENTAL MODEL

We recently explored the mechanism of adaptation against the novel antiangiogenic TKIs, nintedanib and dovitinib. The key finding was that the agents that acted to correct the abnormal physiology of tumour blood vessels and to correct hypoxia ultimately led to a major switch in cancer metabolism (Navarro *et al*, 2016). The high glucose uptake that is inherent to most epithelial malignancies (especially in MAPK- and/or Pi3K-AKT-activated tumours), which is also known as the Warburg effect, is needed not only to satisfy energy requirements but also to supply additional carbon skeletons required by an anaplerotic shift in the Krebs cycle (Lunt and Vander Heiden, 2011). Our data showed that although glycolysis was restricted in the presence of antiangiogenic TKIs, because of changes in HIF1-α and AMPK signalling, the tumours continued growing normally. This finding suggests that under selective pressure, tumour plasticity led to sustained tumour growth over the long term. However, under these conditions, the tumours were dependent on the continuous uptake and degradation of fatty acids and ketone bodies, which are targeted for mitochondrial catabolism. This switch to an alternative metabolic source became essential for tumour survival. When several breast and lung cancer models were 'primed' with normalising antiangiogenic TKIs, the use of mitochondrial
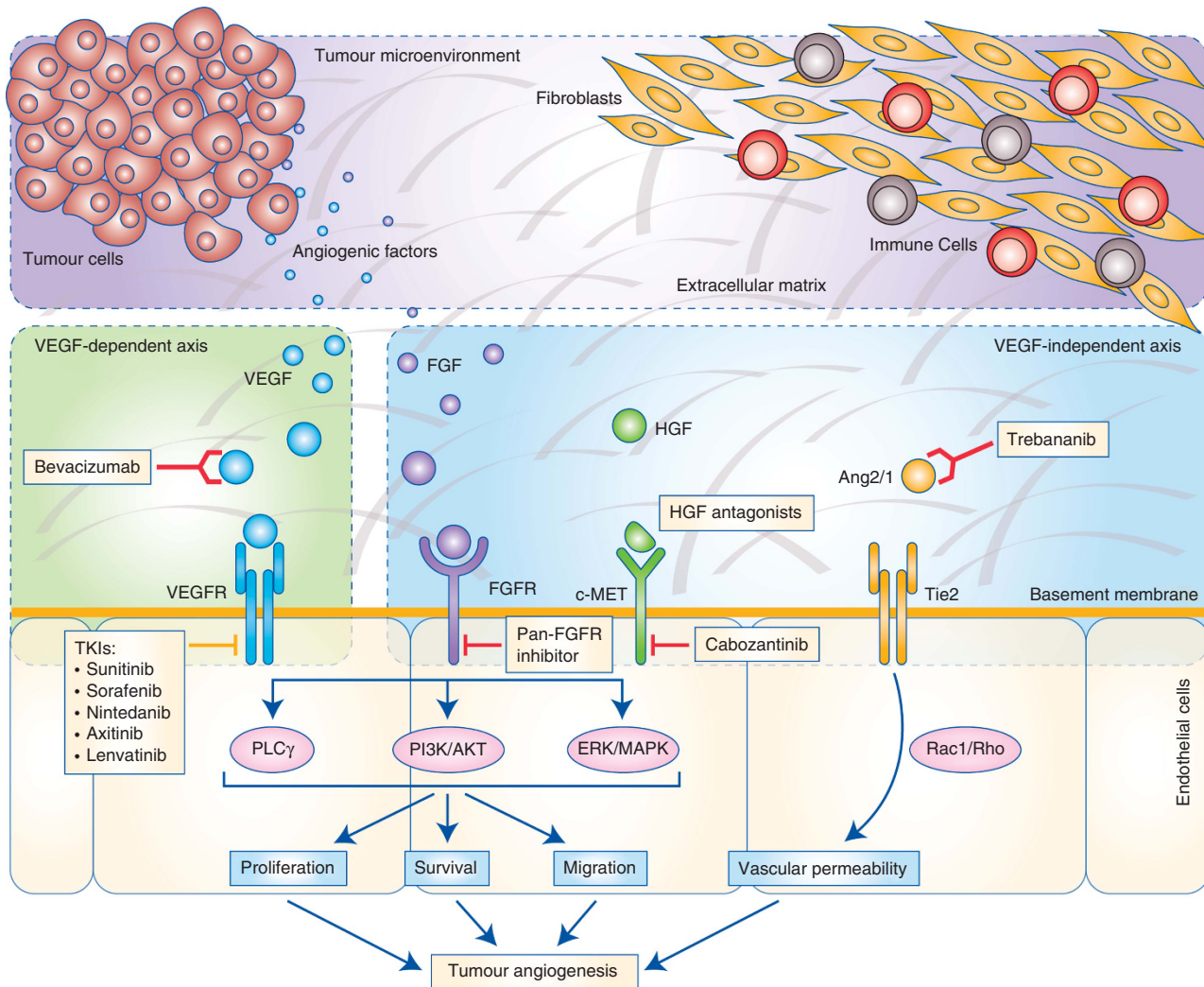
Figure 1. Tumour angiogenesis: molecular pathways and potential interventional strategies. VEGF axis is recognised as the primary factor responsible for tumour angiogenesis resistance. Upon VEGF binding, the receptor tyrosine is activated. VEGF/VEGFR activates oncogenic signalling via mitogen-activated protein kinase (ERK/MAPK) pathway, the phosphotidylinositol 3 kinase (PI3K-AKT) pathway, and the phospholipase-C-γ (PLC-γ) pathway leading to cell proliferation, survival, migration, and vascular permeability. Chronic VEGF/VEGFR inhibition has been found to lead to the emergence of compensatory signalling pathways that sustain angiogenesis. Several of these VEGF-independent signalling pathways, rely on key proteins, including the FGF/FGFR, angiopoietin-2 (Ang2), and the MET oncogene. Complementary therapies focused on these alternative pathways have been developed to abrogate acquired resistance. Pan-FGFR inhibitors are used to inhibit the FGF/FGFR axis. The MET/HGF pathway is inhibited by HGF antagonists and by the anti-MET antibody cabozantinib. Ang-Tie axis can be targeted with trebananib, a peptibody that inhibits the binding of Ang1/2 to their receptor. Tumour microenvironment and interactions between tumour cells and non-malignant cells as fibroblast and leukocytes also affect angiogenesis by interfering with the signalling pathways required for cell recruitment and vascular construction.

inhibitors (phenformin or ME-344) led to impressive antitumour effects. The dependence on mitochondrial metabolism was only evident in this situation, as the antimitochondrial agents were inactive when used in monotherapy. This point is of clinical relevance because of the rising interest in biguanides in the medical community. Biguanides have been associated to lower cancer burden in diabetic patients (Evans *et al*, 2005). However, according to preclinical data, biguanides can exert a direct effect in cancer cells but also an indirect effect by correcting the pro-cancerous metabolic-inflammatory aberrations that exist in diabetic patients (Foretz *et al*, 2014). The fact that metformin requires the presence of a membrane transporter to enter cancer cells (but not phenformin; Sogame *et al*, 2009) together with the observation that phenformin shows more potent anticancer effects than metformin (Yuan *et al*, 2013) suggest that the ideal agent to test in prospective clinical trials in patients not selected by being

diabetic would be phenformin. Nonetheless, a recent phase III trial adding metformin to standard chemotherapy in pancreatic cancer did not show meaningful benefits of this intervention (Kordes *et al*, 2015).

In addition, studies of tumour cells indicated that our findings did not represent an autonomous response to TKIs, as none of the reprogramming events involving signalling pathways, transcriptomics, and metabolomics were observed *in vitro*. These findings suggest that the metabolic switch requires the interaction of several specialised cell lineages in the tumour microenvironment. We have termed this phenomenon 'metabolic synthetic lethality'. Our work considers that the tumour behaves 'as a whole' and that the phenotype is a reflection of the largest proportion of cells in the tumour (Figure 2). Obviously, our model does not imply that 100% of the cells within a tumour undergo the same type of reprogramming, but as long as the epithelial cell compartment
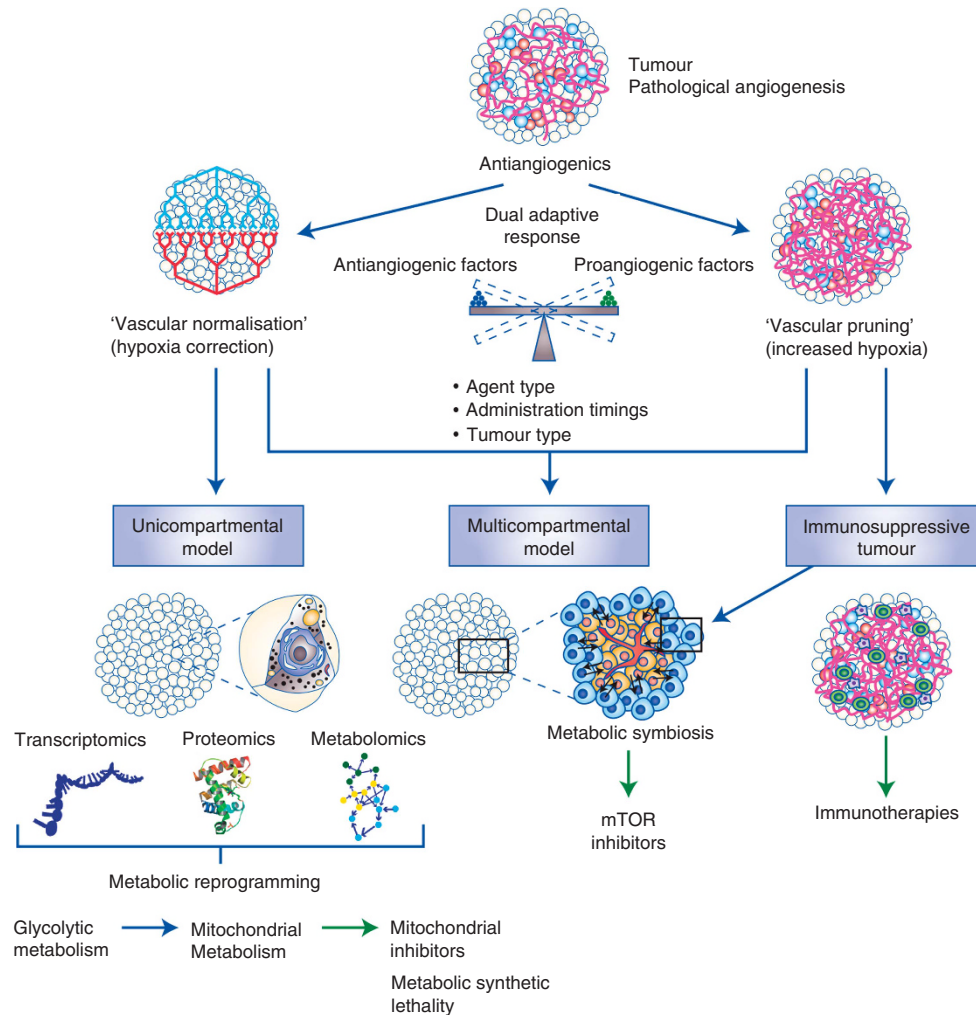
Figure 2. Dual microenviromental adaptive response against antiangiogenic therapies and its clinical implications. Tumours can exhibit a dual different adaptive response, vascular 'normalisation' (associated to hypoxia correction) or vascular pruning (associated to increased hypoxia), that would be determined by the type of antiangiogenic agent, dynamic changes in the concentrations of pro- and antiangiogenic factors, administration timing, and tumour type. Vascular normalisation corrects oxygenation leading to a metabolic switch. This metabolic reprogramming requires the interaction of several specialised cell lineages in the tumour microenvironment and it is characterised by an increased in dependence on mitochondrial metabolism. Upon this situation tumours become vulnerable to mitochondrial inhibitors inducing the phenomenon of metabolic synthetic lethality. The process may not be homogeneous: some tumour areas may experience hypoxia correction and others hypoxia increase. In this case, an alternative model proposes a metabolic compartmentalisation of tumours. In hypoxic regions cells import and metabolise glucose, whereas in normoxic regions tumour cells activate lactate catabolism leading to the upregulation of mTOR signalling. The resulting upregulation of mTOR signalling could be disrupted by the administration of mTOR inhibitors. Finally, the reprogramming may not be limited to cancer metabolism: vascular pruning and increased hypoxia as response against antiangiogenics can be associated with an immunosuppressive phenotype opening an opportunity for the novel immunotherapies.

makes up a large proportion of the volume of the tumour, the 18F-MISO-PET uptake of epithelial cells can be used to guide therapeutic decisions. However, the work of other investigators, described in the following sections, reveal the reality to be much more complex and probably difficult to fine-tune.

ANTIANGIOGENIC RESISTANCE: MULTICOMPARTMENTAL MODEL

An alternative model for understanding tumour metabolism was described by Lisanti's group as the cooperation between different tumour subpopulations, a model they named the 'reverse Warburg effect' (Pavlides *et al*, 2009). Basically, epithelial cancer cells induce aerobic glycolysis in neighbouring stromal fibroblasts, and the resulting energy-rich metabolites (lactate and pyruvate) are then

transferred to the epithelial cancer cells, where they enter the Krebs cycle, resulting in production of high levels of ATP. Thus, the fibroblastic tumour stroma 'feeds' the cancer cells, representing a type of host–parasite relationship. The shuttle of energy-rich substrates between these cell populations occurs because cancer epithelial cells upregulate their expression of monocarboxylate transporter 1 (MCT1; a lactate importer) and induce the expression of MCT4 (a lactate exporter) in fibroblasts (Whitaker-Menezes *et al*, 2011). Cancer cells induce transformation of normal stroma by secreting hydrogen peroxide, which triggers oxidative stress in nearby fibroblasts, resulting in reduced mitochondrial activity and increased glucose uptake (Martinez-Outschoorn *et al*, 2011). This metabolic compartmentalisation was shown for subcompartments of tumour epithelial cells as well. Sonveaux *et al* (2008) demonstrated that compartmentalisation of tumour cells was a particular form of adaptation to hypoxia. In hypoxic

regions, cells import and metabolise glucose by glycolysis, with upregulation of the expression of the glucose importer GLUT1 and MCT4. In normoxic regions, tumour cells activate oxidative metabolism; they express the lactate importer MCT1 and avidly import lactate anions and glutamine. Therefore, a metabolic symbiosis, which can be disrupted by inhibiting one of the components such as the MCT1 transporter in the symbiotic pathways, allows the mutual survival of these two regions (Sonveaux et al, 2008). Metabolic symbiosis was recently examined in different tumour models, and found to be associated with acquired resistance to antiagiogenics (Allen et al, 2016; Jimenez-Valerio et al, 2016; Pisarsky et al, 2016). In response to the vascular collapse and consequent hypoxia induced by antiangiogenic agents, cancer cells are compartmentalised based on their occurrence in hypoxic or normoxic regions, which depend on distance from the few remaining functional blood vessels. Under these conditions, hypoxic cancer cells induce the expression of GLUT1 and MCT4, and normoxic cells express the lactate transporter MCT1. Moreover, the metabolic pathway of lactate catabolism increases glutamine metabolism, leading to the upregulation of mammalian target of rapamycin (mTOR) signalling (Allen et al, 2016). The resulting metabolic symbiosis and upregulation of mTOR signalling in normoxic cells could be disrupted by the concomitant administration of antiangiogenic agents and rapamycin or everolimus, translated in significant antitumor effects (Allen et al, 2016; Jimenez-Valerio et al, 2016). All of these findings underscore the fact that tumour heterogeneity and plasticity are the main barriers to achieving durable responses with targeted agents used as monotherapy. The occurrence of a unicompartmental or multicompartmental response may depend on the type of agent administered and whether its effects lead to global or spatial normalisation. Investigating the reprogramming events occurring in each tumour subpopulation may help clarify the mechanisms of resistance and result in rational, tailored, synergistic regimens for those patients who develop acquired resistance against antiangiogenics agents (Figure 2).

ANTIANGIOGENIC RESISTANCE IN THE CONTEXT OF INCREASED HYPOXIA: IMMUNE REPROGRAMMING

A critical step in tumour progression is the ability of tumour cells to evade immunosurveillance by the immunocompetent host. Antiangiogenics can correct or increase hypoxia, and this can be limited to certain tumour areas or in the whole tumour volume. Regardless of the extent of the antiangiogenic-induced hypoxia, in this context the 'reprogramming' may not be limited to tumour metabolism. Emerging data indicate that tumour hypoxia is related to an immunosuppressive phenotype associated with the upregulation of the transcription factor HIF1-α. An immunosuppressive microenvironment is promoted by HIF1-α by recruiting regulatory T cells, and increasing the expression of programmed death-ligand 1 (PD-L1) in tumour cells and myeloid-derived suppressor cells. Moreover, the growth factors and cytokines (e.g., transforming growth factor β and VEGF) induced by hypoxia suppress the activity of T lymphocytes and inhibit the ability of dendritic cells to process tumour antigens and present them to lymphocytes (reviewed in Kumar and Gabrilovich, 2014; Palazon et al, 2014). Preclinical observations have already suggested that hypoxia correction by antiangiogenics are associated to correction of the immunosuppressive environment as well (Huang et al, 2012). To date, the published evidence about the opposite situation (antiangiogenics that increase hypoxia because of vascular pruning) is scant; our ongoing (unpublished) research suggests that immune reprogramming could be an interesting focus of research on methods that enhance the activity of immunomodulatory

agents. However, the steps leading to adaptation events and their targetability remain to be elucidated.

CLINICAL IMPLICATIONS

The findings we have discussed are important because of their immediate clinical applicability. To induce the phenomenon of metabolic synthetic lethality for patients with early breast cancer, we have already launched a phase I trial that combines bevacizumab treatment with weekly doses of ME-344 started 1 week after the first dose of antiangiogenic agent. A second trial investigating the combination of phenformin with nintedanib for patients with lung or colon cancer will start at the end of the year. Finally, a third investigator-initiated trial is exploring the effects of adding anti-PD-L1 treatment to durvalumab for patients with advanced breast cancer who progressed while on bevacizumab maintenance treatment. On the basis of the recently published study investigating lenvatinib and everolimus (launched before the metabolic symbiosis hypothesis was tested at the preclinical level; Motzer et al, 2015), other trials are exploring the addition of mTOR inhibitors for patients with kidney cancer.

However, optimising the findings, that is, applying the right treatment combination (immune or metabolic modulators) at the individual level, requires the investigation of two remaining questions. First, we must confirm that the pattern of stromal adaptation can be detected in the metastatic setting. In our preoperative study, we only included patients with early breast cancer and single lesions (Quintela-Fandino et al, 2016). We cannot rule out the hypothesis that in the metastatic setting, heterogeneous adaptive patterns might be found, with some tumour lesions showing increased and others showing decreased hypoxia. Such a scenario would complicate clinical decision making. Second, although we showed how corrected or increased hypoxia correlated, respectively, with treatment efficacy or lack of it, we have not yet demonstrated in the clinical setting that the addition of a metabolic inhibitor or an immunomodulator is associated with enhanced therapeutic effects. In addition, a quantitative assessment of peripheral blood specimens would be preferable to imaging tests for guiding therapeutic decisions. Ongoing research for peripheral blood biomarkers is focused on hydroxylated proteins.

Angiogenesis is a complex process, and understanding the mechanisms of resistance in order to optimise therapy seems to depend both on the mechanism of action of the specific agent under study and the tumour type, which requires assessment of each individual patient. Novel lines of research on resistance to antioangiogenic agents extend beyond the study of the implications of several signalling axes and point towards both the cooperation between several cell lineages and metabolic reprogramming. Personalised decision making may be possible by incorporating novel hypoxia/normoxia biomarkers with recent discoveries on dual adaptive patterns of resistance and may prolong the time that metastatic patients benefit from a class of drugs for which precision medicine has been elusive.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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